AD______________

GRANT NUMBER DAMD17-94-J-4363

TITLE: Oligosaccharide Markers for Prognosis of Low-Risk Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. David E. Pettijohn

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Denver, Colorado 80262

REPORT DATE: September 1996

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
September 1996

3. REPORT TYPE AND DATES COVERED
Annual (1 Sep 95 - 31 Aug 96)

4. TITLE AND SUBTITLE
Oligosaccharide Markers for Prognosis of Low-Risk Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4363

6. AUTHOR(S)
Dr. David E. Pettijohn

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado Health Sciences Center
Denver, Colorado 80262

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

During the period since the last progress report, the expression of several different cell surface oligosaccharides and proteases have been defined on sections from 86 different ductal breast carcinomas. Monoclonal antibodies specific for the blood group related oligosaccharides Le^a, Le^x, sialyl- Le^a, Le^y, Tn, Sialyl Tn and extended Le^a- Le^x were used to define the cell surface oligosaccharides and both immunofluorescence techniques and immuno streptavadin-biotin techniques were used to evaluate the fraction of tumor cells that are positive and the relative amount of reaction product as measured using an image processing system. The preliminary analysis of the available data has shown that the extended Le^a-Le^x oligosaccharide is a statistically significant indicator of poor prognosis following surgical removal of small, node-negative ductal breast carcinomas. cDNA libraries constructed from cells that express Le^a- Le^x have been screened and selected for cDNAs that alter the expression of this oligosaccharide in cancer cells. Two candidate genes have been identified and preliminarily characterized.

14. SUBJECT TERMS
Breast Cancer prognosis, oligosaccharides, carbohydrates, glycoproteins immunofluorescence, monoclonal antibodies

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

_X_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

_X_ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

_X_ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

David E. Pettijohn 9-27-96

PI - Signature Date

TABLE OF CONTENTS

ANNUAL REPORT FOR GRANT NUMBER DAMD17-94-J-4363

Front Cover	1
SF 298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	5
Conclusions	7
References	7
Appendix	9
Bibliography	11

INTRODUCTION

Prognosis is generally favorable for breast cancer patients who have a small node-negative ductal carcinoma (see for review ref. 1). Relapse during a 5-10 year period after surgery occurs in less than 20% of these so called low-risk patients. However, oncologists are faced with a difficult decision in managing such patients, as there is no clear way of identifying the 20% who will relapse. Thus the patients who could benefit most from adjuvant chemotherapy cannot be identified and equally important, the patients who don't require post-surgical adjuvant therapy cannot be unambiguously identified. The purpose of the present research is to devise an approach for identifying the low-risk patients who are at risk.

The general goal of these studies is to determine if there are specific combinations of oligosaccharide markers and other makers on breast cancer cells that are useful in predicting the post surgical prognosis of low-risk node-negative breast cancer patients. Useful prognostic markers identified from these studies would then be combined with other known prognostic markers in an attempt to assemble a set of markers which could indicate with highest specificity and sensitivity the patients who are at greatest risk for relapse. The studies are also intended to identify a new glycosyltransferase activity that seems to be expressed in certain carcinomas and is correlated with poor prognosis. This identification would open the way for new approaches to studying the biological effects of the significant oligosaccharides that are correlated with poor prognosis.

We are studying a large group of breast tumor specimen obtained from a collection of the Danish Breast Cancer Cooperative Group which is a nationwide surveillance and research program (2). All specimen are from women who had low-risk node negative ductal breast carcinomas and who had surgery 5-15 years previously and who have been closely followed since surgery. None of the women had chemotherapy, so that the prognosis is unaffected by other post-surgical interventions. A panel of well characterized monoclonal antibodies with known specificity for specific oligosaccharides is employed to define the cell surface oligosaccharides, proteolytic activities (such as Cathepsins) and protease inhibitors associated with the tumor cells. After completing the analysis, the relapse history of the patients will be compared with the different molecular markers using Cox's proportional hazards model to identify statistically significant independent markers of prognosis. It will then be possible to select different combinations of markers to attempt to improve specificity and sensitivity by using a panel of prognostic markers.

Additional related research is seeking to identify the glycosyltransferase activities that are abnormally expressed in breast cancer cells that lead to aberrant expression of specific marker oligosaccharides. Here we are attempting to clone cDNAs recognizing genes that are expressed in cells overexpressing the Le^a-Le^x oligosaccharide, which is the best prognostic indicator which we have identified. We are also beginning studies of the effects of Le^a-Le^x cell-cell interactions in carcinomas.

This research is still in progress and was planned to be in progress at this stage. Therefore conclusions and detailed summaries of the data to date are premature. However the preliminary review of the data provided below indicates that there is a statistically significant association of the Le^a-Le^x oligosaccharide and poor prognosis of low-risk ductal breast carcinomas.

BODY

We continue to use the panel of monoclonal antibodies (Mabs) specific for the designated oligosaccharides (Table 1) and in the last year have applied the complete panel to multiple paraffin sections of the first 86 tumor specimen to be examined. As in the previous year, we used double-label immunofluorescence microscopy techniques that apply fluorescene and rhodamine conjugated antibodies simultaneously so that the distribution of two different oligosaccharides can be simultaneously determined in the same tumor section (3,4). The Quantimet 500+ Image Processing System was used to analyze fluorescence images and to define both the fraction of tumor cells that are positive, (above a defined baseline), and the intensity of the reaction relative to positive and negative control cells that are processed at the same time. The

fraction of positive tumors cells and the relative amounts of each cell surface components on the tumor cells is therefore determined.

As reported in the last progress report, we continue to observe significant heterogeneity for these oligosaccharides among the cells in certain tumors. For example Mab 43-9F recognizing the extended Le^a-Le^x oligosaccharide reacts with nearly 100% of the cells of a few breast carcinomas and about 30% of other carcinomas are completely negative, but the majority of the carcinomas have a fraction of cells that are positive, ranging from 1 to 100 % of the cells in a section. We are now beginning to understand some of the reasons for this heterogeneity and recently published a paper offering part of the explanation (5). We found that the expression of many oligosaccharides on the cell surface of cancer cells is dependent on the interactions with adjacent cells. For example, some tissue culture cells such as NU6-1 cells (that we are using to clone the genes coding for critical glycosyltransferases -see below) express large amounts of cell surface Le^a-Le^x oligosaccharide when the cells grow touching neighbors in small colonies or in confluence layers, but express no detectable Le^a-Le^x when cells are attached to substrata and growing without close neighbors. Other cell surface oligosaccharides appear when cells grow at low densities, but extinguish or are less plentiful when cells reach confluence. Experiments completed in the past month showed that the critical cross-talking between cells is dependent on cell type -- for example, cells of other carcinomas will not substitute for NU6-1 cells in inducing expression of Le^a-Le^x.
Thus it seems that the prognostic markers that we study inform us about more than just the status of the individual cells in tumors, but also convey information about the cell-cell signaling within the tumor.

During the past year our Danish collaborator Dr. Johann Andersen encountered personal and professional problems, resigned his position at Odense University Hospital and retired. Because of these problems he stopped communicating with us, and later, after retiring, informed us that he would not be able to provide more breast tumor specimen. It has taken several months for Dr. Andersen's successor to be named and for us to re-establish appropriate contacts. Dr. Carsten Rose, Department of Oncology, Odense University Hospital has informed us that he will keep the commitments to supply the remaining specimen that are required for this project. Thus we will be able to complete the study, but due to the lack of tumor specimen, have fallen behind in the study of Le^a-Le^x. We have made up some ground by competing the study with the entire panel of MABS (Table 1) using the available specimen, and believe that we can nearly catch up with the schedule once specimen are delivered from Dr. Rose.

Statistical analysis of the 86 specimen that have been competed are in progress now at the Biostatistics Core Laboratory of the University of Colorado Cancer Center. We are analyzing both single markers, multiple markers in combinations, and attempting a protocol for the analysis of the ratios of makers in attempts to sharpen prognostic indications of the multiple markers. As noted in the last progress report, the preliminary analysis of the Le^a-Le^x marker alone showed statistically significant ($P<.005$) correlation with poor prognosis. The statistical analysis is using the proportional hazards model of Cox (6).
Thus we have not completed Task 1 and are behind schedule there, but have completed a significant part of tasks 2, 3 and 4.

The project designed to clone cDNAs specifying the glycosyltransferase required to extend Le^a into Le^a-Le^x oligosaccharides (Task #5) is progressing. As noted in the last report the p-bluescript cDNA library made from total mRNA of the human lung cancer cell line NU6-1 (which overexpresses the Le^a-Le^x oligosaccharide (3) was subjected to subtractive hybridization against a similar library made from cDNA homologous to mRNA of the NU6-1 variant clone NE-18 (that makes no detectable Le^a-Le^x). This yielded 3 clones that were of interest among the many that were screened. Two clones have cDNA sequences of unidentified genes, and as previously noted the third clone has the DAF gene (Decay Accelerating factor). These then correspond to mRNA plentiful in a cell line that makes Le^a-Le^x and absent in a close variant cell line that makes no detectable Le^a-Le^x. These genes have been transfected into several different cell lines and we have seen in several cases dramatic changes in the expression of cell surface oligosaccharides. One of the unknown genes, called SIL, drastically reduces the amount of Le^a-Le^x on NU6-1 cells, while increasing the positivity using MABS 19-9 (sialyl-Le^a) and CO514 (Le^a). In other cell lines such as Ne-18, HT-29, T47D, and COS-1, other surface epitopes are affected (Table 2). The DAF

gene codes for a glycosyl-phosphatidylinositol anchored surface molecule that is expressed on the cell surface of malignant tumors (7-10). We found that this gene transfected into different cell types has the general effect of reducing surface oligosaccharide expression, except in HT-29 cells where Le^a-Le^x and other oligosaccharides show increased expression (Table 2). Other breast cancer cell lines MDA-453, MDA-468, and MCF-10A are currently being transfected with the SIL and DAF genes.

It is desirable to target glycosyltransferases in a more specific manner than is possible with subtraction libraries. For this reason we constructed a library derived from NU6-1 cells in the Lambda ZAP Express Vector (Stratagene). This vector allows expression in both prokaryotic and eukaryotic systems. The new library will be normalized (11) and then transfected into cell lines that show negativity for MAB 43-9F (Le^a-Le^x), but are positive for other cell surface oligosaccharides. Cells that express Le^a-Le^x will be isolated after binding MAB 43-9F using magnetic beads coated with goat anti-mouse IGM antibody. This should select cells that have acquired the ability to synthesize Le^a-Le^x. The plasmid will be isolated from these cells, transformed back into bacteria, grown up and repurified, and subjected to additional rounds of transfection into mammalian cells and re-selection. Transfections will be done using standard commercially available reagents {Lipofectamine (Gibco) LT1(Panvera). Toxicity testing and the determination of optimal conditions for transfection in the selected cell lines have already been accomplished using a beta-gal reporter gene.

CONCLUSIONS

Results to date continue to support the conclusion that the prognosis is poorer when low-risk small ductal breast carcinomas are positive for extended Le^a-Le^x oligosaccharide. The more complete testing of this possibility will await the accrual of more data. oligosaccharide. Appropriate cell-cell interactions among tumor cells capable of expressing the extended Le^a-Le^x oligosaccharide are required for expression and interactions with adjacent cells incapable of expression will not substitute. The SIL and DAF cDNAs cloned in this research are candidates for essential factors in the synthesis of extended Le^a-Le^x in cancer cells

REFERENCES

1. McGuire, W.L. and G.M. Clark. 1992 Prognostic factors and treatment decisions in auxiliary node-negative breast cancer. N. Engl. J. Med 326: 1756-1761.

2. Ottesen, G.L., H.P. Graversen., M Blichert-Toft, K. Zedeler, and J. Andersen. 1992 Ductal carcinoma *in situ* of the female breast: Short-term results of a prospective nationwide study. Amer. j. Surg. Pathol. 16: 1183-1196.

3. Pettijohn, D., O. Pfenninger, J. Brown, R. Duke and L. Olsson. 1988. Tumorigenic human squamous cell lung cancer cells have cell surface carbohydrates that are absent from nontumorigienic cells. Proc. Natl. Acad. Sci. USA 85: 802-808.

4. Stranahan, P., R. Howard,. O. Pfenninger, M. Cowen, M. Johnson, and D. Pettijohn. 1992. Mucin gel formed by tumorigenic squamous lung carcinoma cells has Le^a-X oligosaccharides and excludes antibodies from underlying cells. Cancer Res. 52: 2923-2930.

5. Stranahan, P., LaRoe, J., McCombs, R., Goldsmith, A., Rahim, I., Overland, M. and D. Pettijohn. 1996. Cell-cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins. Glycoconjugate J. 13: 1-7.

6. Cox, D. R. 1972. Regression models and life tables. J. Royal Soc. B 34: 187-220.

7. Koretz, K., S. Bruderlein, C. Henne and P. Moller. 1992. Decay-accelerating factor (DAF, CD55) in normal colorectal mucosa, adenocarcinomas and carcinomas. Br. J. Cancer 66: 810-814.

8. Hadam, M. 1989. Cluster Report CD55. *In Leucocyte Typing IV; White Cell Differentiation Antigens.* Eds. Knapp, W., Dorken, B., Gilks, W., Rieber, E., Schmids, R., Stein, H and von dem Borne, A. p 694-97. Oxford Univ. Press.

9. Cheung, N., Walter, E., Smith-Mensah, W., Rainoff, W., Tykocinski, M.,and Medof, M. 1988. Decay-accelerating factor protects human tumor cells from complement-mediated cytotoxicity *in vitro*. J. Clin. Invest. 81, 1122-1128.

10. Terachi, T. Stanescu, G., Pontes, J., Medof, M., and Caulfield., M. 1991. Coexistence of autologous antibodies and decay-accelerating factor, an inhibitor of complement, on human renal tumor cells. Cancer Res. 51, 2521-2523.

11. Soares, M., Bonaldo, M de F., Jelene, P., Su, L. Lawton, L. and Efstratiadis, A. 1994. Construction and characterization of a normalized cDNA library. Proc. Natl. Acad. Sci., 91, 9228-9232.

APPENDIX

Table 1. Oligosaccharides Studied in This Investigation

Oligosaccharide Abreviation	Structure	Monoclonal Antibody
Le^a	Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1--- 4 ↓ Fucα1	CO-514
Sialyl- Le^a	NeuAcα2→3Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1--- 4 ↓ Fucα1	NS19-9
Le^x	Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 3 ↓ Fucα1	P12
Le^y	Fucα1→2Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 3 ↓ Fucα1	F-3
Le^a -Le^x	Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1--- 4 3 ↓ ↓ Fucα1 Fucα1	43-9F
Tn	Galβ1→3GalNAcα1→O--Ser/Thr	IE3
Sialyl-Tn	NeuAcα2→Galβ1→3GalNAcα1→O--Ser/Thr	TKH2
Tx	Galβ1→4GalNAcα1→O--Ser/Thr	3C9

TABLE 2

% of Cells Positive for Specific Monoclonal Antibodies Before and After Transformation by SIL or DAF

Cell Lines	Mab 43-9F	Mab CO514	Mab P12	Mab 19-9	Mab IE3	Mab TKH2	Mab HH8
NU-6--1	50%	30%	Neg	85%	Neg	Neg	Neg
NU-6-1-SIL	1%	50%	30%	90%	10%	Neg	Neg
NU-6-1-DAF	10%	10%	Neg	2%	Neg	Neg	Neg
NE-18	Neg	Neg	35%	Neg	10%	Neg	Neg
NE-18-SIL	Neg	Neg	60%	Neg	Neg	Neg	Neg
NE-18-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg
HT-29	10%	50%	2%	50%	Neg	Neg	Neg
HT-29_SIL	80%	80%	10%	80%	Neg	Neg	Neg
HT-29-DAF	80%	80%	2%	80%	Neg	Neg	Neg
T47D	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T47D-SIL	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T47D-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg
COS-1	50%	Neg	Neg	Neg	Neg	Neg	Neg
COS-1-SIL	80%	Neg	Neg	Neg	Neg	Neg	Neg
COS-1-DAF	80%	Neg	Neg	Neg	Neg	Neg	Neg
H157	Neg	Neg	Neg	Neg	Neg	Neg	Neg
H157-SIL	Neg	Neg	Neg	Neg	Neg	Neg	Neg
H157-DAF	Neg	Neg	Neg	Neg	Neg	Neg	Neg

BIBLIOGRAPHY

Stranahan, P., LaRoe, J., McCombs, R., Goldsmith, A., Rahim, I., Overland, M. and D. Pettijohn. 1996. Cell-cell interactions influence oligosaccharide modifications on mucins and other large glycoproteins. Glycoconjugate J. 13: 1-7.

Stranahan, P., Andersen, J., Archer, P., McCombs, R., and D. Pettijohn. 1996. Cancer Associated antigen Le^a-Le^x is predictive for increased risk of disease relapse in low-risk breast cancer. AAAS 87th Annual Meeting Vol. 37 March 1996.

Personnel supported by this grant 1995-96

David Pettijohn, Professor
Patricia Stranahan, Assistant Professor
Iffat Rahim, Research Associate
Ronda McCombs, Professional Research Assistant
Karen Kelly, Assistant Professor
Vern Shellman, Professional Research Assistant
David Well, Student Dishwasher
Doug Tucker, Student Assistant
David Marinace, Student Dishwasher